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Application of Quantitative Proton Nuclear Magnetic Resonance Spectroscopy to Chemical Warfare Agents

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ABSTRACT

This report outlines the development of a quantitative proton NMR spectroscopy method for determining the purity of CWAs using homogeneous internal standards. The method was validated to an accuracy and precision better than 1% through the use of certified NMR standards. The method is useful for determining the purity of major chemical species at concentrations at or above 25 mM, and for identifying and quantifying minor chemical species at or above 0.06 mM and 0.20 mM, respectively. The method was employed to determine the purity of three chemical warfare agents (HD, GB and VX) and was found to be equal to or better than chromatography in terms of precision, accuracy and analysis turnaround time. As qHNMR simplifies the analysis procedure, exposure of personnel and analytical equipment to CWAs is minimised.

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Application of Quantitative Proton Nuclear Magnetic Resonance Spectroscopy to Chemical Warfare Agents

Executive Summary

Proton quantitative Nuclear Magnetic Resonance (qHNMR) Spectroscopy is a non-destructive technique that can be used for quantitation of complex mixtures with absolute errors generally below 2%. This report outlines the development of a qHNMR method for determining the purity of chemical warfare agents (CWAs) using homogeneous internal standards. The method was validated to an accuracy and precision better than 1% through the use of certified NMR standards. The method can be applied to determine the purity of major chemical species at concentrations at or above 25 mM, and to identify and quantify minor chemical species at or above 0.06 mM and 0.20 mM, respectively. The qHNMR method was employed to determine the purity of three chemical warfare agents (HD, GB and VX) and was found to be equal to or better than chromatography in terms of precision, accuracy and analysis turnaround time. The operational simplicity of this method enables quantitative and qualitative information to be rapidly gleaned from a single sample.

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Contents

GLOSSARY

1. INTRODUCTION	1
<i>The Nucleus</i>	2
<i>The Internal Standard</i>	2
<i>Relaxation and Pulse Parameters</i>	2
<i>NMR Data Processing</i>	2
2. RESULTS AND DISCUSSION	2
2.1 Experimental	3
2.1.1 Materials	3
2.1.2 Procedures	3
2.2 Method Validation	4
2.2.1 Linearity	4
2.2.2 Precision and Accuracy	6
2.2.3 Specificity.....	6
2.2.4 Limits of Detection and Limits of Quantitation.....	7
2.3 Determination of CWA Purity.....	8
3. CONCLUSION	12
4. REFERENCES	12

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DSTO-TR-2748

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Glossary

bp	boiling point
CDCl₃	chloroform-d ₁
CWA	chemical warfare agent
FID	free induction decay
GB	sarin (<i>O</i> -isopropyl methylphosphonofluoridate)
GC	gas chromatograph
HD	distilled mustard (bis(2-chloroethyl) sulphide)
LOD	limits of detection
LOQ	limits of quantitation
M	molar (moles per litre)
mg	milligrams
MHz	megahertz (10 ⁶ Hz)
mL	millilitre
mm	millimetre
MS	mass spectrometry
NOE	nuclear Overhauser effect
ppm	parts per million
qHNMR	quantitative proton nuclear magnetic resonance
RSD	relative standard deviation
S/N	signal-to-noise
T₁	spin lattice relaxation time
T₂	spin spin relaxation time
VX	<i>O</i> -ethyl- <i>S</i> -(2-isopropylaminoethyl)methylphosphonothiolate

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1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy sets the standard for solving the molecular structure of aqueous and organic soluble molecules. NMR spectroscopy provides information on the chemical shift (δ), dipolar coupling (J), through-space interactions (nuclear Overhauser effect (NOE)), spin-spin coupling (J) and relaxation parameters (T_1 and T_2).¹ These parameters can be exploited in one or multiple dimensions to provide insight into molecular conformation, reaction kinetics and mechanisms. A fundamental drawback of NMR spectroscopy is poor sensitivity relative to other common laboratory techniques such as gas and liquid chromatography. Despite a lack of sensitivity and high cost of purchase and upkeep, NMR spectroscopy is beneficial as it is a non-destructive technique that enables the direct analysis of complex mixtures. Furthermore, analysis by NMR spectroscopy prevents contamination of laboratory instrumentation and minimises exposure of personnel as samples are fully contained during analysis.

Quantitative proton NMR spectroscopy (qHNMR) has emerged as a powerful technique for quantitation with absolute errors in the range of 0.5-2%.¹ The accuracy of the technique is underpinned by an inherent property of NMR spectroscopy in which the peak area from a fully relaxed spectrum is directly proportional to the number of nuclei giving rise to the peak. Quantitative NMR methods rely on summing all components to 100%, or comparing the integrated peak area of an analyte with that of a reference. The latter approach requires calibration, which is achieved by addition of an internal, external or electronic standard.² Today, most qHNMR methods rely on the addition of a known amount of internal standard. In order to determine the purity of an analyte, the analyst must know the weighed mass and molecular weight of the analyte and the internal standard as well as the number of protons giving rise to the peaks selected for quantitation (at least one peak each for the internal standard and analyte). The purity of the analyte P_x is given by Equation 1:

$$P_x = \frac{I_x}{I_{std}} \times \frac{N_{std}}{N_x} \times \frac{M_x}{M_{std}} \times \frac{m_{std}}{m_x} \times P_{std}$$

Equation 1.

where M_x and M_{std} are the molar masses of the analyte and the standard, m_x and I_x correspond to the weighed mass and integrated signal area of the sample of interest, m_{std} and P_{std} are the weighed mass and purity of the standard, and N_{std} and I_{std} is the number of protons and the integrated signal area of the standard, respectively.³ Using this relationship, Maniara and colleagues showed that qHNMR can be used to determine the purity of organic chemicals with an accuracy equal to or surpassing that of gas and liquid chromatography.⁴ Malz and Jancke reported that under optimised conditions, a qHNMR protocol employing internal standards returns a measurement uncertainty of 1.5% including user-to-user effects. For a qNMR method employing internal standards to be implemented successfully, there are a number of factors that must be taken into consideration, as discussed below.

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The Nucleus

The proton nucleus is frequently used for qNMR applications as it is ubiquitous, yields excellent signal-to-noise, and the acquisition and interpretation of ^1H NMR data is well understood. ^{13}C (1.6% of ^1H sensitivity) and ^{31}P (7% of ^1H sensitivity) are commonly used in qNMR and other nuclei have been investigated.⁵⁻⁹

The Internal Standard

For precise and accurate quantitation, the internal standard must have a known purity, must not react with the analyte, and must have at least one resolved peak in the NMR spectrum. Ideal internal standards are non-volatile, non-hygroscopic and have a limited number of peaks.

Relaxation and Pulse Parameters

During acquisition of qHNMR data, a long interpulse delay is required to re-establish equilibrium z-magnetization prior to application of the next RF pulse. Relaxation rates for protons in small organic molecules are generally in the range of 0.3-5 s, although significantly longer rates have been reported.¹⁰ Methods using a 90° pulse require an interpulse delay of $5 \times T_1$ to ensure full recovery of z-magnetization. In general, an interpulse delay of 60 seconds is more than adequate to ensure that all spin systems have time to re-equilibrate. Alternatively, a pulse at the Ernst angle allows for a reduction of the interpulse delay and thus a higher scan repetition rate and sensitivity.¹¹

NMR Data Processing

A qHNMR spectrum should be carefully processed to obtain spectral characteristics suitable for quantitation. Crucial characteristics include flat baseline, sharp and phased peaks, and absence of spectral artefacts. In order to achieve these objectives, the raw free induction decay (FID) is usually multiplied by an exponential window function with a line-broadening factor prior to Fourier transformation. Chemical shift is referenced to the residual solvent signal or other reference material and then the spectrum is phased and baseline corrected prior to integration.

2. Results and Discussion

The aim of this work was to develop a qHNMR method for determining the purity of chemical warfare agents (CWAs) and cataloguing the identity and concentration of low level species, including degradation products and residual solvents, in CWA standards. This document outlines the evaluation and validation of linearity, accuracy, precision, and specificity parameters but stops short of a complete method validation describing user-to-user effects, as this topic has been dealt with in detail elsewhere.³ Whilst this method would ultimately be applied to the analysis of numerous CWAs, this study focuses on the G-series

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nerve agent sarin, the V-series nerve agent VX and the vesicant agent sulphur mustard (Figure 1).*

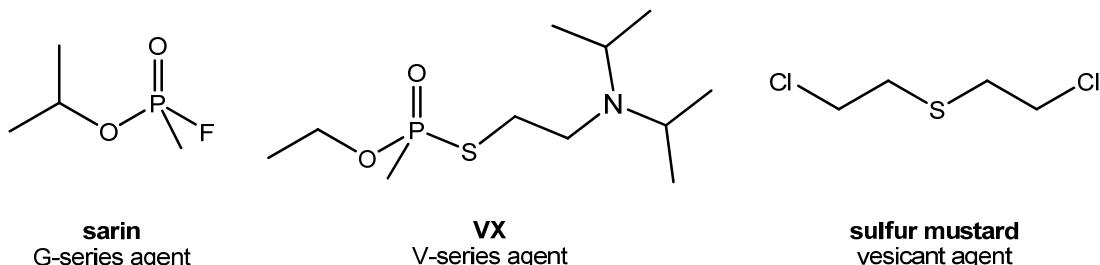


Figure 1. The chemical structure of the CWAs investigated in this work

More than 20 chemicals have been used as reference compounds for validation of qHNMR methods.¹ 1,2,4,5-tetramethylbenzene (δ 6.9 ppm; singlet and δ 2.2 ppm; singlet) and dimethylsulfone (3.0 ppm; singlet) were chosen for the validation of this method. Dimethylsulfone has proven utility as an internal standard in qHNMR¹² and is considered to be particularly versatile as it is soluble in organic and aqueous media. Both of these chemicals are non-volatile crystalline solids and thus well suited for use as internal standards. Volatile solvents are not widely used as internal standards in qNMR due to the likelihood of evaporation during preparation and analysis. However, a valuable sample could easily be recollected simply by evaporation of the internal standard. With this in mind, this study evaluates trichloroethylene, a solvent with a boiling point (bp) of 87 °C and a characteristic singlet at 6.5 ppm in chloroform-d₁ (CDCl₃), as an internal standard.

2.1 Experimental

2.1.1 Materials

Dimethyl sulfone (99.65%, Sigma), 1,2,4,5-tetramethylbenzene (durene) (99.95%, Sigma) and trichloroethylene (99.5%, Sigma) were used in the development of this method. Chloroform-d₁ (D 99.8%, CIL) was used as the deuterated solvent. All solutions are in CDCl₃ unless specified otherwise.

2.1.2 Procedures

A Sartorius CP2245 balance was used for weighing CWAs and a ME5 analytical scale was used for weighing all other chemicals. NMR spectra were acquired on a Bruker Avance Ultrashield 500 MHz spectrometer with a 5 mm BBI Z-GRD probe using Topspin software (V2.1). Table 1 outlines the parameters used for data acquisition.

* GB and VX are racemic mixtures of (+)/(−)-GB and (+)/(−)-VX, respectively.

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Table 1. qHNMR data acquisition parameters

Parameter	Value
Spin rotation	0 Hz
Measurement temperature	298 K
Pulse angle	90°
Preacquisition delay	6.5 us
Acquisition time	3.28 s
Relaxation delay	60 s
Number of scans	8
Sweep width	20 ppm
FID points	64k
Line broadening	0.3 Hz
Frequency of excitation	9 ppm

Raw FID files were multiplied by an exponential window function with a line-broadening factor of 0.3 Hz and then Fourier transformed to give frequency domain spectra. Spectra were referenced to the residual solvent signal, i.e. CHCl₃ for CDCl₃, and the phase and baseline parameters were manually adjusted. Peak integration was extended symmetrically from the peak apex and terminated prior to reaching the ¹³C satellites.

2.2 Method Validation

This section outlines the evaluation and validation of method linearity, accuracy and precision, and specificity parameters. Samples were dissolved in CDCl₃ and the 90° pulse length was determined for each chemical. T₁ relaxation rates for each compound were determined using the inversion-recovery null point pulse sequence with the longest value for each chemical presented in Table 2. None of the chemicals had a T₁ longer than 12 s. Thus, the standard pulse sequence, with an interpulse delay of 60 s, was suitable for validation and purity calculations.

Table 2. T₁ values for CWAs and internal standards in CDCl₃

Chemical	Solvent	T₁ max (s)
HD	CDCl ₃	3.43
GB	CDCl ₃	3.49
VX	CDCl ₃	5.82
dimethylsulfone	CDCl ₃	3.67
durene	CDCl ₃	3.72
trichlorethylene	CDCl ₃	6.07

2.2.1 Linearity

A linear relationship between detector response and sample concentration is required for qHNMR. A series of solutions with 0.1–10 mole of durene per mole of dimethylsulfone as internal standard were analysed to test linearity. The curve presented in Figure 2 demonstrates that a linear relationship between durene concentration and instrument response (integrated peak area) is achieved across the investigated range. Figure 3 illustrates

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that the difference between experimental and the known value between the gravimetric and experimental values vary by less than 1% across the entire range. The linearity test was also performed with trichloroethylene as an internal standard (data not shown). Although linearity was satisfactory over the tested range, the bias between the gravimetric and experimental concentration was found to vary up to 3% in the worst case. The volatility of trichloroethylene can lead to evaporation of the internal standard and overestimation of analyte concentration, which is consistent with the observed effect in this work. Under these conditions, trichloroethylene is not suitable for use as an internal standard and its use was abandoned.

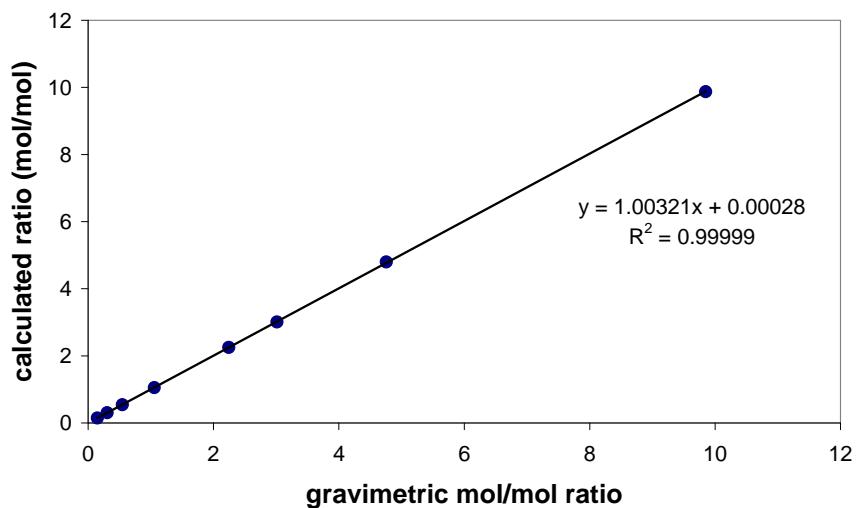


Figure 2. Plot of the known molar ratio of durene and dimethylsulfone compared to the calculated ratio demonstrates that NMR detector response is linear over the tested range. Dimethylsulfone was the internal standard.

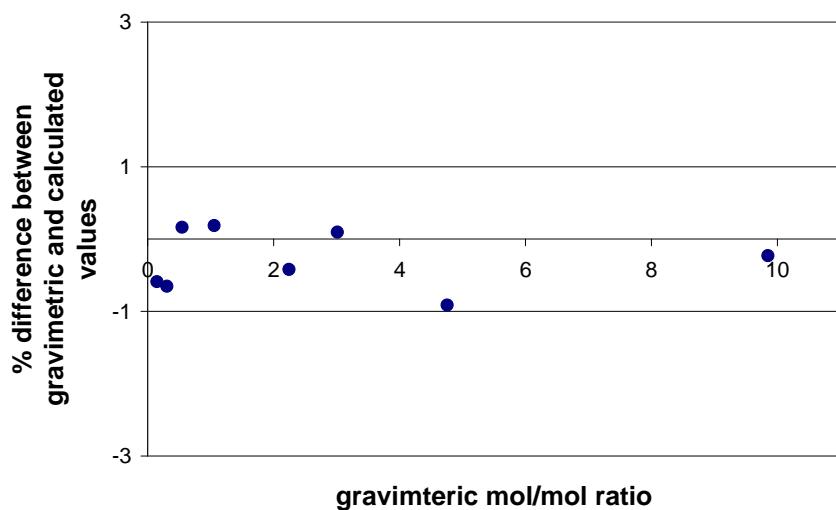


Figure 3. Plot of known molar ratio of durene and dimethylsulfone compared to the difference between the gravimetric and calculated values demonstrates that the ratio does not exceed 1% over the tested range. Dimethylsulfone was the internal standard.

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2.2.2 Precision and Accuracy

In order to develop a useful quantitative technique, we needed to establish the precision and accuracy of this qHNMR method. Accuracy is described by Equation 2:

$$\text{accuracy} = \sqrt{p^2 + b^2}$$

Equation 2.

where p is precision (relative standard deviation (RSD)) and b is bias. In order to evaluate precision and accuracy, a solution of durene (4.324 mg/mL) with dimethylsulfone internal standard was analysed at 25 °C using the standard qHNMR pulse parameters. The purity of durene was calculated according to Eq. 1 and is given in Table 3 below. For data set A, measurement of a single sample was replicated seven times. For data set B, seven individual samples of identical concentration were analysed. For data set A, method accuracy was determined to be 0.4% based on precision and bias values of 0.2% and 0.4%, respectively. In good agreement with these findings, data set B gave a method precision and bias of 0.1% and 0.4%, respectively, giving a method accuracy of 0.4%.

2.2.3 Specificity

Specificity in qHNMR relies upon an analyte that can be quantified without interference from the internal standard or any other compounds in the mixture. It is generally sufficient to analyse the sample of interest prior to addition of internal standard in order to determine if signals from impurities are likely to converge or overlay with the internal standard signal. If overlap or convergence is likely to occur, a new internal standard should be investigated. For complex mixtures, 2D correlation experiments can be used to ascertain whether underlying impurities contribute to the peak area of the analyte signal intended for use in the quantitation process.

A common issue in NMR peak selection is the inconsistent inclusion or exclusion of ^{13}C satellites and spinning sidebands in the integral value. Henderson states that these signals are a component of an actual signal and, therefore, should be included in the integration value.¹³ However, the width of a signal that includes spinning side bands and ^{13}C satellites increases the likelihood of convergence or overlap with impurities, especially in complex samples with crowded spectra. Therefore, it was decided to exclude ^{13}C satellites from the integration value and to prevent the formation of spinning sidebands by not spinning the sample. Importantly, the signals from the analyte and internal standard are treated in the same way; that is, the integration area extends symmetrically from the peak apex and terminates prior to reaching the ^{13}C satellites.

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Table 3.

Durene Purity. ^a Determined by Proton qHNMR Spectroscopy Using Dimethylsulfone Internal Standard at 25°C.		
data set A		
sample	determined purity (wt%)	
1	99.45	
1	99.54	
1	99.39	
1	99.81	
1	99.53	
1	99.34	
1	99.68	
mean	99.53	
RSD	0.17	
data set B		
sample	determined purity (wt%)	
1	99.45	
2	99.72	
3	99.63	
4	99.71	
5	99.55	
6	99.50	
7	99.67	
mean	99.60	
RSD	0.10	

^aactual durene purity $99.95 \pm 0.08\text{wt\%}$; durene solution prepared at $4.324 \pm 0.020\text{ mg/mL}$ in CDCl_3

2.2.4 Limits of Detection and Limits of Quantitation

This method is primarily for determining the purity of chemical warfare agents that are not sample-limited. However, it is useful to be able to quantify minor species for cataloguing low level components of interest. To determine the experimental limit of detection (LOD), durene solutions (12.5-100 mM) with DMS internal standard were analysed by qHNMR. The data in Table 4 indicates that solutions above 25 mM are suitable for quantitation as the calculated purity is consistent with the known purity of $99.95 \pm 0.08\text{wt\%}$. Although the signal-to-noise (S/N) value of the 12.5 mM solution exceeds 150:1 as generally required in qHNMR,³ the calculated purity varies greatly from the known value. Analytical methodology generally requires a minimum S/N of 3:1 for LOD and 10:1 for limit of quantitation (LOQ) measurements.¹⁴ Thus, according to the data in Table 4, a 25 mM solution (the lowest concentration deemed suitable for purity determination) will have an LOD of 0.06 mM or 0.2wt% ($25\text{ mM} \times 3/1300$) and LOQ of 0.2 mM or 0.8wt% ($25\text{ mM} \times 10/1300$) for low level components using the standard pulse parameters, assuming the minor components have the same molecular weight and multiplicity as the CWA. It is useful to note that NMR signal

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intensity is proportional to the square root of the number of scans, and thus method LOD/LOQ can be improved by increasing the number of scans when required.⁴

Table 4. A series of solutions with durene concentrations ranging from 12.5-100.0 mM were used to evaluate method LOD and LOQ

Conc ^a (mM)	Purity ^b (wt%)	S/N
12.5	91.28	460
25.0	99.24	1300
50.0	99.27	3600
100.0	100.10	8500

^aConcentration of durene in CDCl₃; ^bDurene purity 99.95±0.08wt%

2.3 Determination of CWA Purity

Seven replicates of CWA (distilled mustard (bis(2-chloroethyl) sulphide) (HD), sarin (*O*-isopropyl methylphosphonofluoridate) (GB), or *O*-ethyl-*S*-(2-isopropylaminoethyl) methylphosphonothiolate (VX)) with internal standard were dissolved in 600 µL of CDCl₃ and analysed by qHNMR spectroscopy. CWA purity was calculated according to Eq. 1 using the integrated peak signals outlined in Table 5. CWA purity values are reported as the mean purity of seven replicates with a 95% confidence interval (mean ± 1.96 standard deviations)

Table 5. Multiplicity and chemical shift of the proton sets used for quantitation. In each case, the aromatic protons of the internal standard (durene; singlet; 6.9 ppm) were used for quantitation.

	Multiplicity	Chemical Shift (ppm)
HD	triplet	3.68
GB	multiplet	4.95
VX	2 × multiplet	4.10 and 4.19

The ¹H NMR spectrum of HD consists of two triplets, theoretically of equal area. However, in each of the seven HD replicates, the triplet at 3 ppm had a larger integral than the triplet at 3.7 ppm by approximately 8%. This is consistent with an impurity peak at 3 ppm contributing to the area of the coincident HD triplet. Based on the chemical shift, the unknown impurity was likely to be 1,4-dithiane, which has been shown to form during storage of HD.¹⁵ Qualitative analysis by GC-FPD-MS confirmed the presence of 1,4-dithiane as a major impurity along with trace levels of bis(2-chloroethyl) ether and bis(2-chloroethyl) disulfide. It is noteworthy that these lower level components were not detected in the qHNMR spectra, highlighting the difference in LOD of the two techniques.

The purity of HD was calculated by qHNMR using the triplet at 3.7 ppm and was determined to be 94.0±0.8wt% as presented in Table 6. 1,4-Dithiane was found to constitute approximately 3% of the sample based on the integral differences of the two triplet signals of the qHNMR

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spectra, as highlighted in the expanded insert in Figure 4.[†] 1,4-Thioxane was detected at a concentration below the LOQ (approximately 0.02wt% of the sample) and was confirmed by comparison to authentic material.

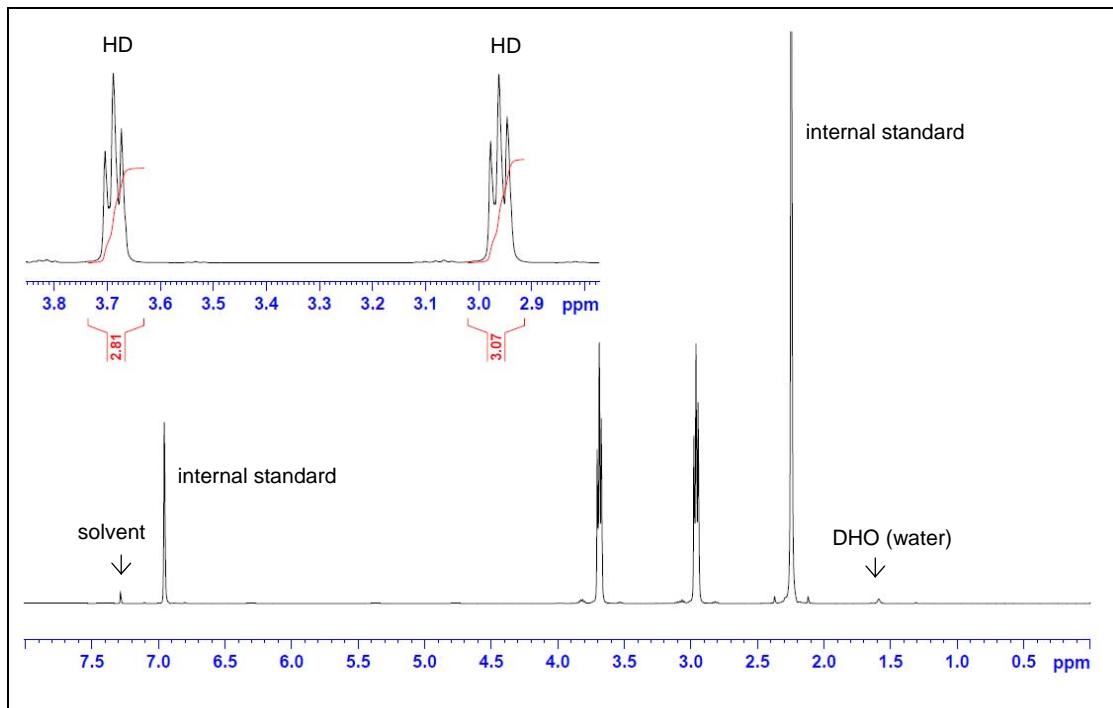


Figure 4. Example NMR spectrum of HD with durene internal standard in CDCl_3

The purity of GB was determined to be $89.1 \pm 1.4\text{wt}\%$ using the integrated peak area of methylylidene multiplet of the O-isopropyl side chain (Figure 5). The sample was found to contain propan-2-ol at a concentration below LOQ (approximately 0.7wt%) and O,O-diisopropyl methylphosphonic acid (DIMP; 6.6wt%), which is a byproduct formed during synthesis.[‡] Finally, the purity of VX (Figure 6) was determined to be $95.4 \pm 1.4\text{wt}\%$ as presented in Table 8.

[†] Concentration of 1,4-dithiane determined using a single replicate

[‡] Concentration of DIMP and propan-2-ol determined using a single replicate

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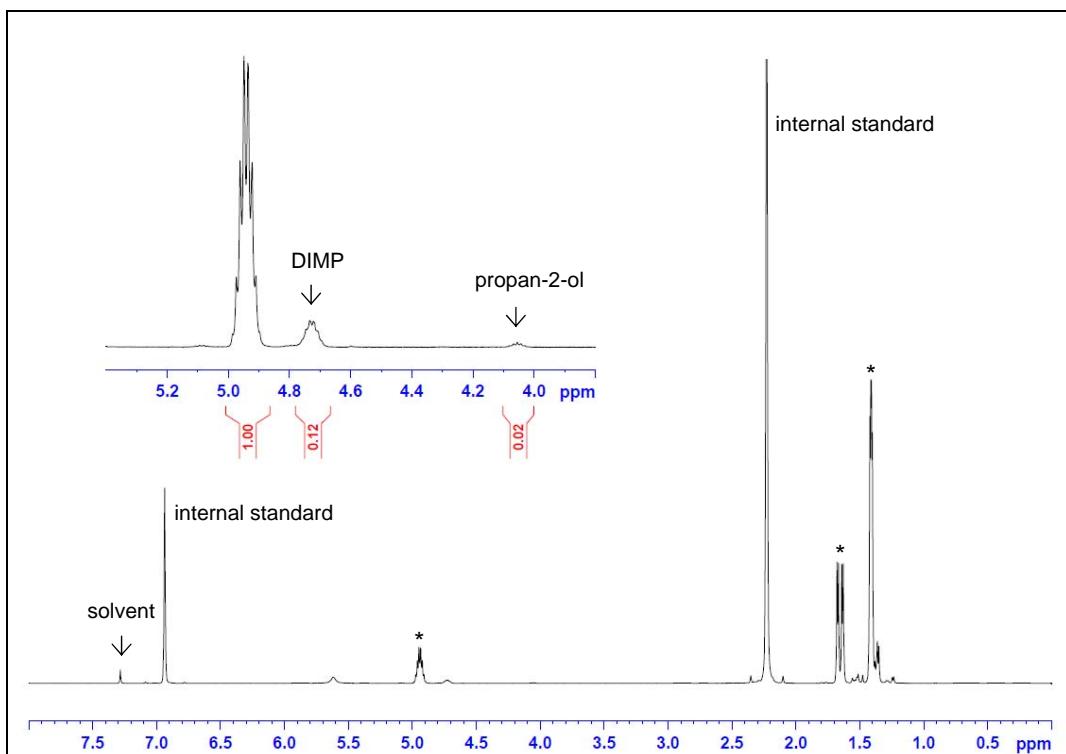


Figure 5. Example NMR spectrum of GB with durene internal standard in CDCl_3 . *Denotes GB resonances. DIMP = O,O -diisopropyl methylphosphonic acid.

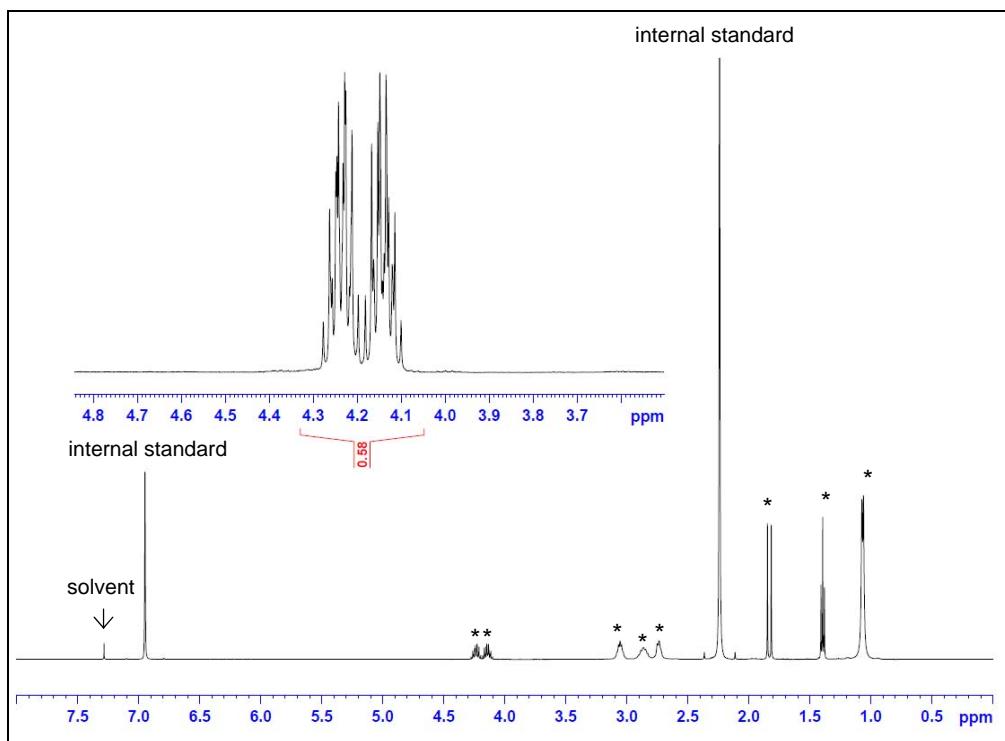


Figure 6. Example NMR spectrum of VX with durene internal standard in CDCl_3 . *Denotes VX resonances.

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Table 6. The purity of HD was determined by qHNMR spectroscopy using seven replicates

HD purity	
replicate	purity (wt%)
1	93.9
2	93.6
3	94.0
4	94.7
5	94.3
6	94.1
7	93.7
mean	94.0
RSD	0.4

Table 7. The purity of GB was determined by qHNMR spectroscopy using seven replicates

GB purity	
replicate	purity (wt%)
1	89.4
2	89.7
3	88.0
4	89.3
5	88.2
6	89.3
7	89.6
mean	89.1
RSD	0.8

Table 8. The purity of VX was determined by qHNMR spectroscopy using seven replicates

VX purity	
replicate	purity (wt%)
1	96.0
2	94.4
3	96.2
4	95.8
5	95.2
6	95.6
7	95.4
mean	95.4
RSD	0.7

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3. Conclusion

This report outlines the development and evaluation of single pulse ^1H NMR spectroscopy method for the accurate and precise determination of CWA purity. Using a set of defined acquisition parameters, a sample containing multiple analyte targets can be quantitatively measured by comparison to a single homogeneous internal standard. The primary benefits of this approach include: (i) the minimisation of separation chemistries; (ii) the reduced consumption of solvents and consumables; (iii) full containment of samples during analysis; and (iv) the non-destructive analysis of samples. The operational simplicity of this method allows quantitative and qualitative information to be rapidly gleaned from a single sample.

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19. ABSTRACT This report outlines the development of a quantitative proton NMR spectroscopy method for determining the purity of CWAs using homogeneous internal standards. The method was validated to an accuracy and precision better than 1% through the use of certified NMR standards. The method is useful for determining the purity of major chemical species at concentrations at or above 25 mM, and for identifying and quantifying minor chemical species at or above 0.06 mM and 0.20 mM, respectively. The method was employed to determine the purity of three chemical warfare agents (HD, GB and VX) and was found to be equal to or better than chromatography in terms of precision, accuracy and analysis turnaround time. As qHNMR simplifies the analysis procedure, exposure of personnel and analytical equipment to CWAs is minimised.				